PROXIMAL GUT MUCOSAL EPITHELIAL HOMEOSTASIS IN AGED IL-1 TYPE I RECEPTOR KNOCKOUT MICE AFTER STARVATION

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Abstract

Background—Previous studies have shown that starvation induces small bowel atrophy, and that atrophy diminishes with aging. In this experiment, we assessed whether starvation-induced atrophy of proximal gut mucosa is associated with the Interleukin-1 receptor (IL-1R) signaling pathway in aged mice.

Materials and Methods—Thirty 26-month-old IL-1R knockout mice and age-matched wild-type C57BL/6 mice were randomly divided into two groups: *ad libitum* fed and fasted. Mice were euthanized 12 or 48 hours after starvation. The proximal small bowel was harvested for morphologic analysis. Gut epithelial cell proliferation was detected using immunohistochemical staining for proliferating cell nuclear antigen (PCNA), and apoptosis was identified using terminal deoxyuridine nick-end labeling (TUNEL) staining.

Results—Aged IL-1R knockout mice were larger than aged-matched wild-type mice ($p<0.05$). Proximal gut mucosal height and mucosal cell number were not different between aged IL-1R knockout and wild-type groups. The apoptosis index in gut epithelial cells was higher in fed IL-1R knockout versus wild-type mice ($p<0.05$), while no significant difference in cell proliferation between both groups. Mucosal atrophy was induced in both aged IL-1R knockout and wild-type groups by starvation ($p<0.05$), however, aged IL-1R knockout mice experienced greater losses in proximal gut weight, mucosal length, and corresponding cell number than did wild-type mice at the 12-hour time point ($p<0.05$). The apoptosis index in gut epithelial cells significantly increased

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in both groups after starvation (p<0.05). Starvation decreased cell proliferation in IL-1R knockout mice (p<0.05), but not in wild-type mice.

Conclusions—The response in aged IL-1R knockout mice differs from wild-type mice in that starvation increases atrophy and is associated with decreased cell proliferation rather than increased apoptosis.

Keywords

Small intestinal epithelia; apoptosis; proliferation; TUNEL staining; PCNA staining

INTRODUCTION

Epithelial cell turnover in the small intestine represents a balance between cell proliferation and cell death. This constant replacement of cells slows with aging, resulting in decreased absorption of proteins, carbohydrates, and fatty acids, and leading to an inability to maintain nutrition via enteral support [1-4]. While nutritional deficiencies in the elderly can be corrected by nutritional supplementation [5-7], especially among patients who are fed enterally [8-10], there is still a need to determine whether age-induced changes in the integrity of the gastrointestinal tract result in diminished function. Previous studies indicate that age-related changes do result in gut dysfunction. Starvation has been shown to cause proximal gut atrophy with increased epithelial cell apoptosis and decreased cell proliferation in adult mice [11,12]. We have observed that aged mice have distinct hallmarks of gut epithelial homeostasis as well as higher apoptotic rates in the gut epithelium. Starvation-induced proximal gut mucosal atrophy was also diminished in aged mice, and was associated with less decreased epithelial proliferation [13]. However, a mechanistic link demonstrating how starvation alters the balance between gut epithelial cell apoptosis and proliferation is still unclear.

Starvation initiates physiological and metabolic changes, including suppression of immune function [14-16] and immune signaling molecules such as IL-2 in fasted patients [17]. Alteration of the immune response may subsequently lead to gut mucosal epithelial homeostasis [18]. Interleukin-1 (IL-1) was reported to affect both cell death and survival in gut epithelia via the IL-1 receptor (IL-1R) [19-21]. There are two forms of IL-1R: type I and type II. The type I receptor is primarily responsible for transmitting the inflammatory effects of IL-1 while type II receptors may act as a suppressor of IL-1 activity by competing for IL-1 binding. By using the IL-1R type I knockout mouse model, our group has determined that proximal gut epithelial homeostasis was altered, with increases in both cell proliferation and apoptosis, indicating that IL-1 might play a pivotal role of maintaining gut epithelial homeostasis in adult mice [22].

In this study, we examined the effect of starvation on proximal gut mucosal homeostasis in aged IL-1 type I receptor knockout mice. Aged mice expressed distinguishable characteristics of gut mucosal homeostasis and responded differently to starvation. We, therefore, hypothesize that IL-1 beta is associated with regulating starvation-induced stress signal transduction in the proximal gut mucosal epithelium.

MATERIALS AND METHODS

Thirty 26-month-old male IL-1R knockout mice and age-matched wild-type C57BL/6 mice (Harlan Sprague-Dawley, Houston, TX) were housed in individual cages in a temperature controlled cubicle with a 12-hour light-dark cycle. Knockout mouse genotypes were verified by polymerase chain reaction to confirm the absence of the IL-1R allele [23]. Mice were fed a regular laboratory diet for one week to acclimatize them to our facilities. The study was

approved by the Animal Care and Use Committee of the University of Texas Medical Branch, Galveston, Texas.

All of the mice were randomly divided into fed and fasted groups, and were housed individually in cages with wire-grid floors to prevent scatophagy during the experimental period. The fed groups (n=10) had access to chow and water *ad libitum*, while the fasted groups (n=20) had access to water only (no chow). Mice were sacrificed by decapitation 12 or 48 hours after removal of food. The entire small bowel was excised, weighed and divided into halves. The first 1-cm segment from the proximal end of the small bowel was opened longitudinally and immediately immersed in 10% buffered formalin for further histological and immunohistochemical analysis. The subsequent 1-cm segment was weighed (proximal gut wet weight), baked at 50°C for 3 days, and then weighed again (dry weight). Dry and wet gut weights were used for gross estimation of gut mass.

Mucosal morphology

Formalin-fixed tissues were processed and embedded in paraffin. Mucosal morphology was examined in 4-μm thick hematoxylin and eosin histologic sections. Villus height and crypt depth were measured using 10 consecutive villi at 10X magnification with a calibrated eyepiece on a light microscope. An observer, blinded to animal treatment, also counted mucosal cell number in the same villi. An average value was calculated for each animal.

Immunohistochemistry staining

Apoptotic cells were identified using the TUNEL (terminal deoxyuridine nick end labeling) immunohistochemical method (Apoptag; Oncor, San Francisco, CA) [24]. 3-μm thick sections were deparaffinized, rehydrated in graded alcohol soaks (100%, 95%, and 70%), and washed with deionized water. Protein was digested using proteinase K (20 μL/mL in phosphate buffered saline [PBS]). Endogenous peroxidase activity was quenched with 2% H_2O_2 in PBS. Each section was treated with 75 μl of equilibration buffer, followed by diluted TdT enzyme solution and incubated at 37°C for 1 hour. Slides were then incubated with 55 μL of anti-digoxigenin peroxidase at room temperature for 30 minutes. Color development was achieved by applying diaminobenzidine-H_2O_2 to the tissue sections. Six sections of each tissue block were obtained at 40-50 μm intervals and stained. In each section, TUNEL positive cells were counted in10 fulllength villi. Apoptotic epithelial cells were identified as those exhibiting brown nuclear staining or apoptotic bodies, which are fragments of apoptotic cells engulfed by neighboring epithelial cells. Intra-epithelial lymphocytes were excluded based on morphologic examination. All epithelial cells within the villi and crypt were counted. For each of the six sections, the epithelial apoptosis rate was expressed as the percentage of apoptotic cells among the total cells in the section. The percentage of apoptosis for the proximal gut of each animal was calculated by averaging the values for all six histologic sections.

Proliferation was quantified using proliferation cell nuclear antigen (PCNA) (DAKO CORPORATION, CA) staining. Deparaffinized and rehydrated sections were incubated with PCNA-horseradish peroxidase conjugate at a 1:50 dilution overnight at 40°C. The sections were then washed with PBS, and color was developed using diaminobenzidine-H2O2, with hematoxylin as a counterstain. PCNA-positive cells were counted on six sections from each animal. The cell proliferation index was expressed as the percentage of PCNA-positive cells among the total cells counted from the base of the crypt to the villus tip.

Statistical analysis

Data were analyzed by one-way ANOVA with Tukey's test or unpaired Student's t-test where appropriate. Significance was accepted at $p<0.05$. Data are expressed as means ± SEM.

RESULTS

Proximal gut epithelial homeostasis in aged IL-1 receptor knockout mice

Aged IL-1R knockout mice were larger than age-matched wild-type mice (36.9±1.6 grams vs. 31.5±0.6 grams; $p<0.05$). The ratios of proximal gut dry and wet weights to body weight were not significantly different between aged IL-1R knockout and wild-type mice. Mucosal morphology, including mucosal height and mucosal cell number, were also not significantly different between groups. However, the apoptosis index for gut epithelial cells was significantly greater in aged IL-1R knockout mice compared to the age-matched wild-type mice ($p<0.05$). There was not significant difference in cell proliferation between IL-1R knockout and wild-type groups (Table 1).

Starvation alters proximal gut epithelial homeostasis in aged IL-1 receptor knockout mice

One IL-1R knockout animal and one wild-type animal died 48 hours following starvation. At 12 hours post-starvation, significant body weight was lost in both the knockout and wild-type groups ($p<0.05$) with no further significant losses observed at 48 hours.

Starvation did not alter the ratio of proximal gut wet weight to body weight in the wild-type group. However, there was a significant reduction in the ratio of proximal gut wet weight to body weight 12 hours after starvation in the IL-1R knockout group ($p<0.05$). The ratio of proximal gut dry weight to body weight decreased earlier in the IL-1R knockout mice than in the wild-type mice ($p<0.05$) (Fig. 1), indicating that starvation may induce rapid activation of the IL-1R signaling pathway.

After starvation, mucosal height and corresponding epithelial cell number decreased significantly in both IL-1R knockout and wild-type groups ($p<0.05$). At 12 hours post-starvation there were significant differences in mucosal length and epithelial cell number between aged IL-1R knockout mice and aged-matched wild-type mice ($p<0.05$) (Fig. 2). These data indicate that starvation-induced mucosal atrophy of the proximal gut is even more pronounced in aged IL-1R knockout mice.

Proximal gut epithelial apoptotic index increased dramatically after starvation in both groups ($p<0.05$) (Fig. 3). PCNA analysis showed a significant decrease in the number of proliferating cells in the IL-1R knockout mice ($p<0.05$), but not in the aged matched wild-type mice (Fig. 4).

DISCUSSION

In this study, we found that starvation-induced proximal gut weight loss was attenuated in aged wild-type mice compared to IL-1R knockout mice, suggesting a role for IL-1R signaling in starvation-induced responses. To identify the mechanism by which weight loss occurred in aged knockout animals, we examined proximal gut epithelial turnover and quantified both cell proliferation and apoptosis. Proximal gut epithelial turnover was not significantly different between IL-1R knockout and wild-type mice, although a higher cell apoptotic rate was noted in aged IL-1R knockout mice. Interestingly, the alteration of proximal gut mucosal cellularity in IL-1R knockout mice is age related. Our group found that both gut epithelial cell death and proliferation increase in adult IL-1R knockout mice

compared to adult wild-type mice [22]. We speculate that physiological changes associated with aging affect gut epithelial cell proliferation rather than cell apoptosis following blockade of the IL-1 receptor signaling pathway, thereby implicating the role of IL-1R in cell proliferation.

In the current study, we found that proximal gut atrophy was greater in aged IL-1R knockout mice than in wild-type mice 12 hours after starvation. Ratios of proximal gut wet and dry weight to body weight, mucosal length, and epithelial cell number decreased more in IL-1R knockout mice compared to wild-type mice. These findings suggest that IL-1R mediates the atrophic response to starvation and is active in maintaining proximal gut mucosa.

During the aging process progressive organ deterioration is accrued, affecting both structure and function [25]. Our previous studies using aged mice showed that starvation-induced atrophy of proximal gut mucosa decreased with decreased reduction in cell proliferation [13]. In the current study, we have shown that starvation increased gut epithelial cell apoptosis in both aged IL-1R knockout and wild-type mice. However, there is a significant decrease in cell proliferation only in aged IL-1R knockout mice. Increased atrophy in proximal gut mucosa is associated with decreased cell proliferation in aged IL-1R knockout mice, again supporting a role for IL-1R in maintaining cell survival.

Small intestinal epithelial homeostasis is regulated by cytokines and chemokines [26,27]. Gut mucosa atrophy decreased in T-cell receptor knockout mice following a burn injury; this decrease is associated with partial blockade of TNF activation [28]. Interleukin-1 (IL-1) is known to maintain or increase immune cell viability [29,30]. In the current study, we revealed a novel role of IL-1 receptor signaling in response to starvation in aged mouse gut epithelial cells. This finding may provide important mechanistic approach regarding intestinal cell dysfunction in the elderly.

Starvation causes mucosal atrophy and loss of mucosal height [32], and glutamine starvation has been shown to induce apoptosis through specific caspase activation in rat intestinal epithelial cells [31]. While starvation is known to stimulate both epithelial cell apoptotic and proliferation pathways in adult mice and rats [33,34], we have demonstrated that intestinal cell proliferation is less sensitive to starvation in aged mice [13]. In the current study, we found that in aged IL-1R knockout mice, cell proliferation decreased significantly in response to starvation. Based on the aged fasted mouse model, this finding suggests that IL-1 beta plays an important role in cell survival.

In summary, starvation induced increased atrophy of the proximal gut mucosa in aged IL-1R knockout mice. However, by blocking the IL-1 type I receptor, downstream signal transduction pathways associated with epithelial homeostasis are altered in response to starvation, resulting in decreased cell proliferation. These findings suggest that the IL-1R plays a pivotal role in cell survival following external stress on the gut mucosa, and this result might lead to a new therapeutic approach related to improve clinical outcomes in elderly patients.

Acknowledgments

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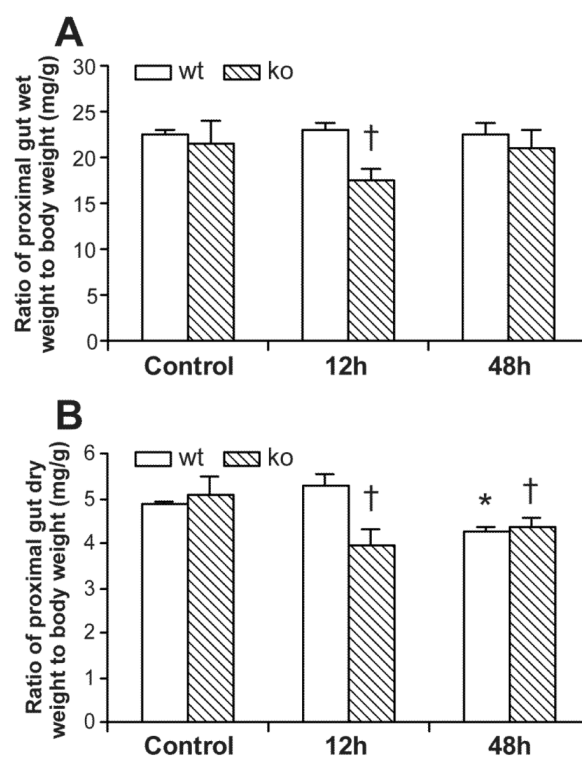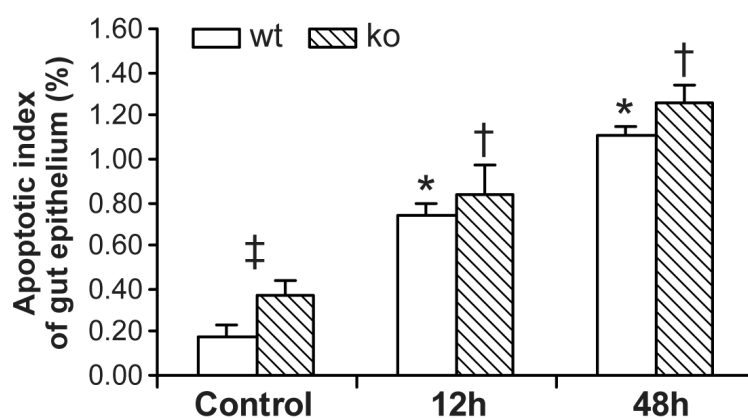
Figure 1.
The ratio of (a) proximal gut wet weight to body weight after starvation and (b) proximal gut dry weight to body weight after starvation. †, $p<0.05$, aged knockout (ko) mice after starvation vs. normal feed control group; *, $p<0.05$, age-matched wild-type (wt) mice after starvation vs. normal feed control group.
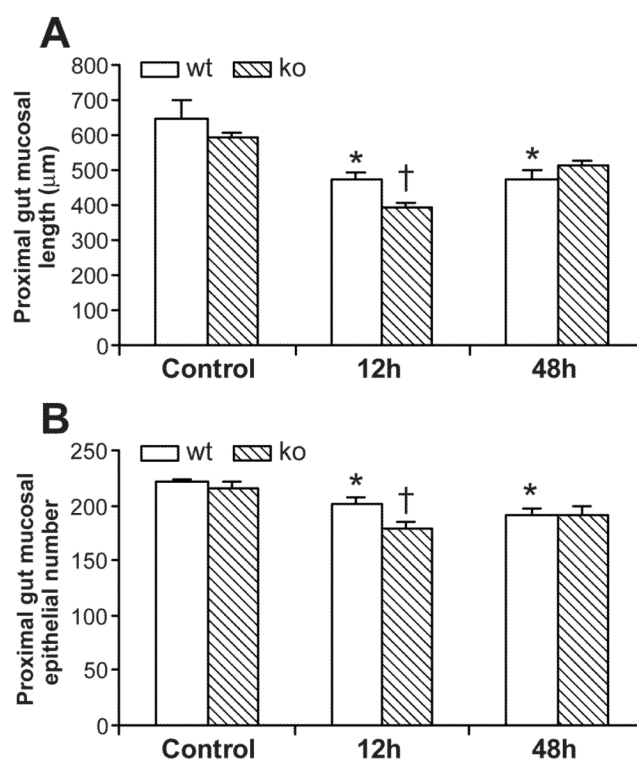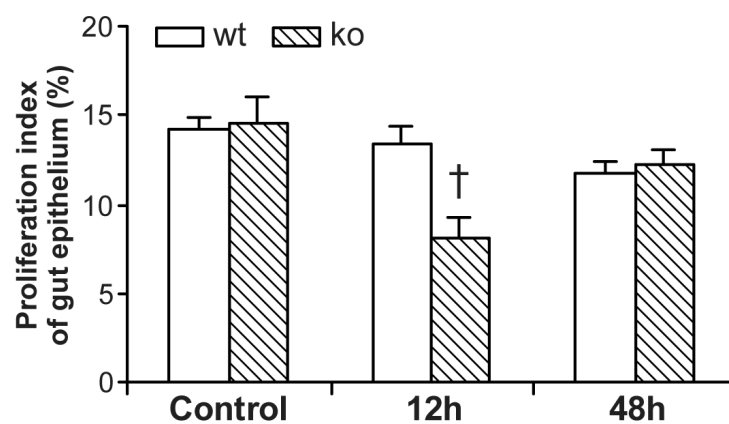
Figure 2.
The alteration of proximal gut mucosal epithelial morphology (a) mucosal length (b) mucosal epithelial number after starvation. †, p<0.05, aged knockout (ko) mice after starvation vs. normal feed control group; *, p<0.05, age-matched wild-type (wt) mice after starvation vs. normal feed control group.

Figure 3.
The apoptotic index of proximal gut mucosal epithelial with TUNEL staining. †, p<0.05, aged knockout (ko) mice after starvation vs. normal feed control group; *, p<0.05, age-matched wild-type (wt) mice after starvation vs. normal feed control group; #, p<0.05, aged knockout (ko) mice vs. age-matched wild-type (wt) mice at each time point.

Figure 4.
The proliferation index of proximal gut mucosal epithelial with PCNA immunohistochemistry staining. †, p<0.05, aged knockout (ko) mice after starvation vs. normal feed control group.

Table 1

Gross changes in proximal gut mucosa and gut epithelial homeostasis between non-starved, aged IL-1R knockout and age-matched wild-type mice.

	wt	ko
Body weight (g)	31.5±0.6	36.9±1.6 [*]
Proximal gut wet weight ratio	22.6±0.5	21.6±2.3
Proximal gut dry weight ratio	4.9±0.1	5.1±0.4
Gut mucosa length (mm)	649±49	591±17
Epithelial cell number	221±3	216±6
Cell proliferation index	14.28±0.54%	14.53±1.55%
Cell apoptotic index	0.18±0.05%	0.37±0.07% [*]

[*], p<0.05, aged knockout (ko) mice vs. age-matched wild-type (wt) mice.